Sustained Decrease in Superoxide Dismutase Activity Underlies Constrictive Remodeling After Balloon Injury in Rabbits

Paulo F. Leite, Alexandre Danilovic, Patricia Moriel, Katia Dantas, Stefan Marklund, Ana Paula V. Dantas, Francisco R.M. Laurindo

Objective—The redox pathophysiology of vascular repair is incompletely understood. We assessed the role of vascular superoxide dismutase (SOD) activity in oxidative/nitrative stress and caliber loss postinjury (PI).

Methods and Results—Rabbits submitted to iliac artery balloon overdistension were followed for 14 days PI. Significant decrease in vascular SOD activity occurred at 7 and 14 days PI (by 45% and 34%, respectively, versus control, 96 ± 1 U/mg, P < 0.05). Separation in concanavalin-A column showed that both extracellular SOD (ecSOD) and CuZn SOD activities were reduced, whereas Western analysis showed normal or augmented protein expression. Immunoreactivity to nitrotyrosine, neuronal NO synthase (NOS), and inducible NOS (iNOS) increased in media and neointima PI; iNOS mRNA also augmented. Administration of ecSOD from days 7 to 14 PI corrected the SOD activity decrease and minimized caliber loss by 59% (P = 0.007) despite unaltered neointima. Nitrate levels markedly increased with ecSOD in injured artery homogenates (26 ± 5 versus 4 ± 0.3 μmol/L per mg, P = 0.001). Such increase was 70% inhibited by specific iNOS antagonist 1400W. Nitrotyrosine and neuronal NOS expression decreased after ecSOD.

Conclusions—Sustained low vascular SOD activity has a key role in constrictive remodeling after injury, promoting oxidative/nitrative stress and impairment of iNOS-derived NO bioavailability. SOD function may critically determine whether iNOS induction is beneficial or deleterious in vivo. (Arterioscler Thromb Vasc Biol. 2003;23:2197-2202.)

Key Words: superoxide dismutase ■ oxidative stress ■ nitrotyrosine ■ nitric oxide ■ vascular remodeling

Vascular repair reaction after injury is characterized by neointimal growth as well as vascular remodeling, which is the main determinant of the final lumen caliber.1 Such events are not only underlying contributors to restenosis after angioplasty but also a relevant model of pathophysiological mechanisms shared by other vascular diseases, such as atherosclerosis, hypertension, and diabetes mellitus.1 One common denominator of such processes is oxidative stress and the associated decrease in NO bioavailability.2,3 Accordingly, studies from our laboratory and other laboratories suggest that redox processes play a role in the signaling program of the vascular repair reaction.4 In particular, reactive oxygen species (ROS) production is prominent immediately after injury and underlies nuclear factor-κB activation4 as well as several other signaling targets.4 ROS production in neointima and adventitia has been documented at later stages of vessel repair.6 Consistent protective effects, particularly in the antagonism of constrictive remodeling, have been demonstrated with the antioxidant probucol.7,8 However, the molecular physiology of redox pathways after injury is incompletely understood. Previous studies focused on the sources of ROS production, suggesting a role for NAD(P)H oxidase immediately after injury as well as after neointima formation.2,9 In particular, there is increased expression of the NAD(P)H oxidase subunits nox-1, nox-4, and gp91-phox in the rat carotid neointima.2 In contrast, less attention has been given to the role of antioxidant enzymes. We observed previously that at 7 days after rabbit iliac artery injury, the response of lucigenin reductase activity to the superoxide dismutase (SOD) inhibitor DETC was indirectly suggestive of low SOD activity.7 SODs, and particularly extracellular SOD (ecSOD), are major regulators of NO bioavailability in vessel wall.10 After injury, inducible NO synthase (iNOS) mRNA is known to be upregulated,11 but it is unclear whether such response is protective or leads to formation of deleterious reactive species. In the present study, we examined in detail the occurrence of SOD low activity and its pathophysiological role in vascular repair and caliber. In addition, we explored the in vivo interaction between SOD activity, nitrative stress, and NO/NOS system.

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Methods
This study complied with the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, rev. 1996) and was approved by a local committee.

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From the Laboratory of Vascular Biology (P.F.L., A.D., P.M., K.D., A.P.V.D., F.R.M.L.), Heart Institute (INCOR), University of São Paulo School of Medicine, São Paulo, Brazil, and Umea University (S.M.), Sweden, and Unicastelo (P.M., K.D.), São Paulo, Brazil.
Correspondence to Francisco R.M. Laurindo, MD, Vascular Biology Laboratory, Heart Institute (INCOR), University of São Paulo School of Medicine, Av. Enés de Carvalho Aguiar, 44, subsole, CEP 05403-000 São Paulo, Brazil. E-mail expfrancisco@incor.usp.br

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Balloon Injury and Vascular Caliber Analysis

Right iliac artery overdistention injury was performed in pentobarbital-anesthetized rabbits, as described previously, using a coronary angioplasty–type balloon with diameter of 3.0 mm, inflated at 8.0 atm. Vessels were harvested at either 7 or 14 days postinjury (PI) and perfusion-fixed with 4% formalin.

At 7 and 14 days PI, in 14 pentobarbital-anesthetized rabbits, digital-enhanced arteriography of lower abdominal aorta was performed through carotid access. At day 14 PI, arteriography was repeated after intraarterial isosorbide mononitrate (1.5 mg/kg for 5 minutes). Simultaneous imaging of injured right and uninjured control left iliac arteries allowed optimal measurement of injured/rcontrol caliber ratio. In preliminary experiments (n = 6), we showed that absolute caliber (in millimeter) of control left artery remained constant versus baseline at days 7 and 14 PI and that preinjury angiograms showed comparable calibers between right and left arteries. Arteriographic images, recorded in high-quality X-ray films, were divided into 6 segments (please see Figure 1 in the online supplement, available at http://atvb.ahajournals.org, and the caliber of each segment was expressed as the average of 6 equally spaced measurements performed with a manual caliper by 2 blinded observers; interobserver variability was <5%.

Exogenous ecSOD Treatment

Human recombinant ecSOD type C, provided by S. Marklund (Umea University, Sweden), was given from days 7 to 14 PI as 10 mg/kg IV boluses every 48 hours. Dosage was based on the ecSOD half-life and validated previously in rabbits. Recombinant ecSOD specific activity was 116300 potassium superoxide dismutase (KOD) assay units (=3500 traditional units) per mg protein. Control rabbits received saline vehicle. Choice of this time period for ecSOD administration was based on our earlier data showing that 50% of total caliber loss occurs from days 7 to 14 PI. Also, ecSOD treatment was limited to 7 days because of the possibility of hypersensitivity reactions.

Histological Analysis

The injured right iliac arteries were cut into 6 segments matching the arteriographic ones, and 3 to 6 serial sections from each segment were examined. The control left artery was similarly examined in 2 segments. Specimens were embedded in paraffin and stained with the Verhoeff-van Gieson method. Neointimal thickening was expressed as the intima/media area ratio. Collagen density was calculated with the previously described Picrosirius polarization method.

Immunohistochemical Analysis

Monoclonal antibodies were used for NOS isoforms (Transduction Labs), nitrotyrosine (Upstate), and activated macrophages (RAM-11, Dako). Immunohistochemical analysis was performed with standard techniques in frozen vascular tissue using biotinylated secondary antibodies and peroxidase staining. Immunoreactivity to nitrotyrosine and neuronal NOS (nNOS) was assessed 7 days PI both in the absence and 48 hours after a 10-μg/kg IV bolus of ecSOD. Color densitometric analysis was performed with a Leica Quantimet software system.

Vascular SOD Activity and Isoform Separation

Iliac artery homogenates were prepared under liquid N2 and centrifuged at 5000 rpm for 5 minutes. Aliquots of the supernatant were assayed for total SOD activity by monitoring inhibition of the rate of xanthine oxidase–mediated cytochrome c reduction (from Sigma) at pH 7.4, as previously described. Separation of ecSOD and the remaining fractions was performed by chromatography on Con A-Sepharose (Amersham Pharmacia Biotech), as described. SOD activity in each fraction was corrected for protein concentration, assessed with the Bradford technique.

Western Blot Analysis

Western analysis was performed in vascular homogenates normalized for protein concentration (Bradford method) using 12% SDS-PAGE, nitrocellulose membranes, and chemiluminescent ECL detection system (Amersham Pharmacia). Sheep polyclonal antibodies against CuZn or Mn SOD were from Oxis. Goat polyclonal antibody against ecSOD was a kind gift from Russel Bowler, PhD (National Jewish Medical and Research Center, Denver, Colo).

Nitrate Levels in Vascular Homogenates

Vascular homogenates were prepared as above, and 10-μL aliquots were injected into Sievers chemiluminescence analyzer (model 280) with VCl and HCl (at 95°C) as reductants. NO results were normalized for protein concentration. In some experiments, vascular samples were harvested 5 hours after a 2-mg/kg IV bolus of 1400w (aminomethylbenzylacetamide, from Calbiochem). This compound is a specific iNOS antagonist, with selectivity factors of 1:5000 versus eNOS and 1:200 versus nNOS. In some experiments, a 10-μg/kg ecSOD bolus was given concomitantly with 1400w 5 hours before euthanasia.

Reverse Transcriptase–Polymerase Chain Reaction for NOS mRNA

Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of NOS mRNA was performed as described.

Statistical Analysis

Data are mean ±SEM. Comparisons between 2 variables were tested by Student’s t test and among ≥2 variables by one-way ANOVA and Student Newman-Keuls post-hoc test, with SPSS 10.0 (SPSS Inc). Significance level was 0.05.

Results

Sustained Decrease in Vascular SOD Activity After Injury

At 7 and 14 days PI, vascular SOD activity was respectively decreased by 45% (53 ± 11 versus 96 ± U/mg protein for contralateral control; P = 0.005) and 34% (62 ± 9 versus 92 ± 6 U/mg; P = 0.02). By day 28 PI, injured and control vessels exhibited similar normal activities. SOD activity from contralateral control arteries was similar to that observed in iliac arteries from intact rabbits not submitted to any procedure (Figure 1).

In a separate experiment 7 days PI, partial separation in concanavalin A column showed that SOD activity from the (CuZn + MnSOD) fraction was decreased by 37% (P = 0.036) and from the ecSOD fraction by 26% (P = 0.02) versus controls. Average contribution of ecSOD to total SOD activity was 36% in control and 40% in injured arteries (P = NS).

Dissociation Between SOD Activity and Expression After Injury

Western analysis of CuZnSOD showed minor reduction in expression at day 7 but not at day 14 PI (Figure 2). Separate additional experiments in rabbits submitted to injury with a balloon of 2.5 mm in diameter showed no difference in CuZn SOD expression versus control at 7 and 14 days PI, although there was an analogous decrease in SOD activity (not shown). In contrast, ecSOD expression was significantly augmented by days 7 and 14 PI. MnSOD expression was unaltered at 7 and 14 days PI (please see online Figure II). These data show that low activity of SOD isoforms was not accompanied by parallel changes in their expressions.
ecSOD Administration Normalized SOD Activity in Injured Vessels

Administration of ecSOD at 7 days PI prevented decrease in SOD activity in injured arteries, with SOD activity of 92/1000 versus 95/1000/16 U/mg for control (P/1000 NS), which was comparable to normal levels. There was little change in SOD activity in normal vessels.

Exogenous ecSOD Replenishment Minimized Vasocostrictive Remodeling After Injury

At day 7 PI, before treatment randomization to ecSOD or vehicle, injured artery caliber was on average 18% above normal and similar between both treatment groups, indicating comparable degree of inflicted balloon injury. Between days 7 and 14 PI, vehicle-treated rabbits exhibited caliber loss equivalent to 34% of normal diameter, reaching an average caliber of 15% below normal. In contrast, ecSOD-treated rabbits exhibited caliber loss equivalent to 14% of normal diameter, which represents a 59% minimization of lumen constriction (P<0.007) (Figure 3). Diameter of the normal vessels was 2.44±0.11 mm and remained unchanged by ecSOD.

Exogenous intraarterial isosorbide mononitrate administration increased vascular caliber by 30% to 45% in control or injured arteries (not shown) but did not correct the relative decrease in vascular caliber either in saline or in ecSOD-treated rabbits. This indicates that neither overall decrease in caliber after injury nor the effect of ecSOD are attributable solely to vasomotor changes.

Remarkably, ecSOD-induced caliber preservation was independent of neointimal size, which was unaltered (see online Figure III). In balloonized arteries, collagen density is known to correlate positively with constrictive remodeling. Exogenous ecSOD led to an ≈50% decrease in collagen accumulation in the media of injured arteries (see online Figure III). Together, these results suggest that the lumen-preserving effects of ecSOD were attributable mostly to antagonism of constrictive remodeling rather than neointimal growth or vasomotor tone.

Increased Vascular iNOS and nNOS Expression After Injury

Both at 7 and 14 days PI, there was marked increase in iNOS immunoreactivity, particularly in the neointima, which was not observed in normal vessels (Figure 4). Semiquantitative
RT-PCR also showed significant increase in iNOS mRNA by days 7 and 14 PI (see online Figure IV). No clear changes in eNOS immunoreactivity and mRNA (RT-PCR) were detected after injury (data not shown). Macrophages were not immunodetected in significant amounts at 7 and 14 days PI (see online Figure IV), suggesting that at this stage of vascular repair, other neointimal cells were the major sources of NOS.

Notably, immunoreactivity to nNOS was also increased at 7 (Figure 4) and 14 days PI (see online Figure V), as additionally confirmed by Western analysis, which showed a single band at 160 kD. The histological punctate pattern of nNOS expression differed from the more diffuse iNOS pattern.

**Increased Nitrative/Oxidative Stress After Injury Was Prevented by ecSOD**

At 7 and 14 days PI, there was increased nitrotyrosine immunoreactivity in the media and neointima, which was absent in uninjured arteries (Figure 5). Expression of such nitrative/oxidative stress marker was nearly abolished by ecSOD replenishment.

**ecSOD Replenishment Rescued Bioavailable NO From iNOS Activity**

Seven days PI, at baseline, there was no difference in NO$\textsubscript{3}^-$ concentration between injured versus uninjured artery homogenates (Figure 6). However, a marked increase in NO$\textsubscript{3}^-$ was observed after ecSOD administration in injured arteries, whereas in uninjured arteries this increase was much smaller. Treatment with the specific iNOS antagonist 1400w before ecSOD administration inhibited by 70% such increase in NO$\textsubscript{3}^-$ levels. These results suggest that ecSOD replenishment interacted with overexpressed iNOS to elicit a shift toward enhanced preservation of bioavailable NO, which decayed to NO$\textsubscript{3}^-$. 

**ecSOD Inhibited nNOS Expression**

Because NO can downregulate nNOS activity,$^{20}$ we investigated whether ecSOD replenishment might also downregu-
late nNOS expression. Immunoreactivity to nNOS in injured arteries was nearly abolished after ecSOD (Figure 3).

Discussion

We reported evidence that decrease in SOD activity plays a significant role in constrictive vascular remodeling in a rabbit balloon injury model. Replenishment of SOD activity by exogenous ecSOD administration given in the late phases of vascular repair minimized such caliber loss. It is probable, therefore, that low SOD activity has a permissive role in remodeling by allowing oxidative/nitrative stress from sources such as NAD(P)H oxidase and NOS to exert their effects, considering that expression of those enzymes is enhanced during the late stages of vascular repair. Other yet-undetermined sources of ROS might also be involved. Our data suggest that such nitrative/oxidative stress allowed by SOD depletion sustains vasoconstrictive remodeling through an active and potentially reversible mechanism and not solely as a consequence of an early wave of oxidative insult. The concomitant decrease in the activity of both extracellular and intracellular SOD isoforms may represent a concerted action to promote vascular wall repair through suppression of NO-mediated cytostasis and apoptosis, thereby allowing vascular cell proliferation or migration to occur.22

Mechanisms underlying the low SOD activity after vascular injury are yet unclear, but any proposal has to account for the discrepancy between decreased enzyme activity and unaltered or increased protein expression. Considering that SOD dysfunction occurs within a context of vascular cell regeneration following a massive wave of apoptosis soon after injury,23 one can speculate that it may represent a recapitulation of the ontogenetic acquisition of enzyme activity. In developing fetal lungs, ecSOD protein expression confined intracellularly precedes by several days the acquisition of ecSOD activity.24 Another possibility is pathological posttranslational protein modification. Hydrogen peroxide could mediate enzyme inactivation in the context of peroxide activity of either CuZn or ecSOD.25 Given the high concentrations of \( \text{H}_2\text{O}_2 \) needed to inactivate SOD, the physiological importance of this reaction is questionable. However, cofactors such as bicarbonate or carbonate radical might amplify such inactivation.26 Also, the recent demonstration that physiological levels of urate are protective against ecSOD peroxidase activity is an indirect suggestion for this mechanism.26,27 Factors inducing copper loss from SOD could also lead to its inactivation, because copper content correlates directly with enzyme activity.28 Nitration of SOD tyrosine residues is an additional possibility.28 However, although CuZnSOD is known to be susceptible,28 recent observations suggest that ecSOD is significantly resistant to nitration.

Although there was depletion in both cytoplasmic and extracellular SOD after injury, administration of ecSOD alone was sufficient to normalize overall SOD activity and improve vascular caliber. This may be attributable to a particular importance of this isoform or to some overcompensation because of excess ecSOD. In any case, our results indicate that modulation of ROS production and particularly interaction with NO in the extracellular compartment are capable of mediating vascular cell responses to a significant extent. This knowledge adds to the ongoing debate over whether signaling ROS are generated in the intracellular or extracellular space. On the other hand, ecSOD-induced effects might still involve a step of transduction to intracellular redox signals.10

Rather than neointimal growth, ecSOD administration minimized constrictive remodeling, suggesting that the later process is preferentially affected by oxidant stimuli. Previous observations in a pig coronary model showed that vitamins C and E improved vascular wall remodeling rather than neointimal extent.29 Such proposals, however, should be regarded with care, considering that some studies did show decrease in neointimal extent with antioxidants. In addition, lack of ecSOD effect in the neointima in our model may be attributable to the particularly late timing of its administration, after peak proliferation has started. Indeed, a recent study showed that transfection of adenovirus carrying ecSOD after balloon injury in hypercholesterolemic rabbits induced decrease in neointima.31

Improved vascular caliber after ecSOD occurred simultaneously with decreased nitrotyrosine expression and increased nitrate levels. These findings provide insights into mechanisms of in vivo NO-superoxide interaction, although such insights are hardly unequivocal, given the complex pathways suggested in vitro.32 With low SOD activity, the reaction between superoxide and NO generates oxidizing/nitrating species, probably peroxynitrite. Interestingly, peroxynitrite decay to nitrate seems negligible, because, despite increased tyrosine nitration, basal nitrate levels were unaltered in injured versus normal vessels. It is likely that under our in vivo conditions, contrary to in vitro models, peroxynitrite or other intermediates alike react favorably with other accessible targets available at relatively high concentrations, thereby preventing decay to nitrate. After SOD replenishment, the increased nitrate concentration likely reflects a shift of NO-superoxide interaction toward increased NO bioactivity. The decreased collagen content of ecSOD-treated arteries could represent a direct effect of NO or reduced oxidative stress on collagen synthesis or degradation or an indirect effect attributable to NO-induced shift in smooth muscle cell differentiation to a contractile phenotype.34

The significant antagonism of ecSOD-induced nitrate increase by specific iNOS inhibitor 1400W indicates that iNOS is a major source of nitrogen species in this model and a fulcrum of NO-superoxide interaction. The observed increase in iNOS expression by nonphagocytic cells during resolutive stabilization of vascular repair is noteworthy, considering that this isoform has been usually associated with acute inflammation. Although iNOS contributed to nitrative/oxidative stress, our data indicate that a seemingly beneficial activity of NO can be rescued from the enzyme activity by ecSOD replenishment. We propose that the degree of remaining endogenous ecSOD is a major determinant of whether iNOS effect in a given pathological scenario is deleterious or not. Several observations suggest indeed that iNOS effect is not uniformly deleterious.
Increased nNOS expression in the healing vessel is a novel finding of our study. Although regulatory mechanisms of extraneuronal nNOS expression are poorly studied, its increased expression was shown previously in atherosclerosis, possibly as compensatory mechanism for dysfunctional eNOS. Extraneural nNOS function is unknown and might contribute to beneficial ecSOD effects in our model. Remarkably, however, there was decrease in nNOS expression after ecSOD; we speculate that such decrease is connected to enhanced bioactive NO, which is known to inhibit enzyme activity.

In summary, our data suggest that sustained SOD activity decrease significantly contributes to constrictive vascular remodeling after injury. Because ecSOD replenishment minimized caliber loss together with decrease in nitrative/oxidative stress and preservation of iNOS-derived NO bioavailability, low SOD activity may be critical to determine whether increased iNOS expression is deleterious. Our data may imply that antioxidant interventions to prevent negative remodeling might still be effective at resolutive stages of vascular repair. These data may clarify the yet poorly understood pathogenesis of the remodeling process and additionally suggest that ecSOD is a valuable target for gene interventions aimed at minimizing sustained vascular caliber loss, not only after angioplasty, but also in several other conditions.

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